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John Glass
Anthony Yee

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J. Craig Venter Institute
9704 Medical Center Drive
Rockville, MD 20850

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14. ABSTRACT
We are working towards the goal of creating a bacterium that contains only the set of genes that are essential for life. Toward that end, we have conducted transposon mutagenesis experiments, designed to identify genes that are not needed for life under laboratory conditions. We have removed several large elements from the starting genome (M. mycoides JCVI-syn1.0). The reduced genome was successfully transplanted into recipient cells and found to be viable and to grow with a reasonable doubling time. We have begun work on an approach to design and synthesize a minimal genome.

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J. Craig Venter™
INSTITUTE

Construction of a Bacterial Cell that Contains Only the Set of Essential Genes Necessary to Impart Life

Report Title:	R&D Status Report (Quarterly)
Report Number:	HR0011-12-C-0063.1
Reporting Period:	May 17, 2012 to August 16, 2012
Contract No.:	HR0011-12-C-0063
Performing Organization:	J. Craig Venter Institute 9704 medical Center Drive Rockville, MD 20850 USA
Author of Report:	John Glass, Anthony Yee
Principal Investigators:	Hamilton Smith, Clyde Hutchison

Abstract

We are working towards the goal of creating a bacterium that contains only the set of genes that are essential for life. Toward that end, we have conducted transposon mutagenesis experiments, designed to identify genes that are not needed for life under laboratory conditions. We have also removed several large elements from the starting genome (*M. mycoides* JCVI-syn1.0). The reduced genome was successfully transplanted into recipient cells and found to be viable and to grow with a reasonable doubling time. We have begun work on an approach to design and synthesize a minimal genome.

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Summary

Work was initiated on the project on May 17, 2012. The goal of the project is to create a cell that contains the set of genes that are essential for life under ideal laboratory conditions. We are working to minimize *Mycoplasma mycoides* JCVI-syn1.0 (the synthetic version of *Mycoplasma mycoides* subsp *capri*) because

- It has a relatively small genome (1.08 MB).
- It can be readily grown in the laboratory.
- We can chemically synthesize its genome and clone it in yeast as a YCp.
- We can isolate the synthetic genome out yeast as naked DNA and bring it to life by transplanting it into a recipient mycoplasma cell.
- We have developed a suite of tools to genetically engineer its genome.

The initial tasks on the project were successfully completed. The global transposon experiments, using Tn4001-tetM and Tn5-puromycin, have been completed. The results have been incorporated into the transposon map contained in Attachment A.

The 6 IS elements, 6 R-M systems, the ICE element and 7 other large gene clusters from *M. mycoides*-syn1.0 have been deleted using TREC and successfully transplanted into recipient cells.

We have also initiated an effort to design and synthesize a minimal genome.

Introduction

The primary goal of this research is to make a minimal bacterial cell. J. Craig Venter Institute (JCVI) will construct a new strain of the bacterium *Mycoplasma mycoides*, controlled by a genome that contains only essential genes. The minimal cell will (i) define the minimal set of genetic functions essential for life under ideal laboratory conditions, (ii) discover the set of genes of currently unknown function that are essential and to determine their functions, (iii) serve as a simple system for cell modeling, (iv) allow for modularization of the genes for each process in the cell (translation, replication, energy production, etc.) and to design a cell from those modules, and (v) allow more complex cells to be built by adding new functional modules.

The starting point for minimization is the synthetic genome *M. mycoides* JCVI-syn1.0. There are two approaches for minimization:

1. Top down: Start with the full size viable *M. mycoides* JCVI-syn1.0 synthetic genome. Remove genes and clusters of genes one (or a few) at a time. At each step re-test for viability. Only proceed to the next step if the preceding construction is viable and the doubling time is approximately normal.

2. Bottom up: Make our best guess as to the genetic and functional composition of a minimal genome, and then design and synthesize it from oligos. Craig Venter calls this the "Hail Mary" genome.

Methods, Assumptions and Procedures

For both approaches, we need to identify genes that are non-essential and are therefore candidates for removal. We are doing this in three ways:

1. Identify genes with functions that are usually non-essential such as insertion sequence (IS) elements, restriction modification (R-M) systems, integrative and conjugative elements (ICE), etc.

   We have removed the IS elements, R-M systems, and ICE elements using the Tandem Repeat Coupled with Endonuclease Cleavage (TREC) approach, and transplanted the genome into *M.capricolum* recipient cells.

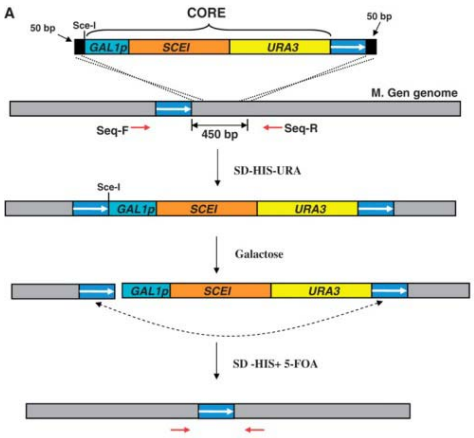
2. Perform global transposon mutagenesis to identify individual genes that can be disrupted without loss of viability.

   We have performed global transposon mutagenesis using two systems, Tn4001-*tetM* and Tn5-puromycin transposomes. The Tn4001 mutagenized genome was sequenced by 454 pyrosequencing and the Tn5 treated genome was sequenced on the Illumina platform. We used the transposon data to create a global transposon map.

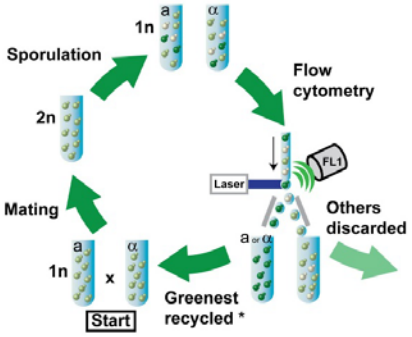
3. Delete targeted individual genes or gene clusters without loss of viability.

   We are using three tools for targeted genome reduction:

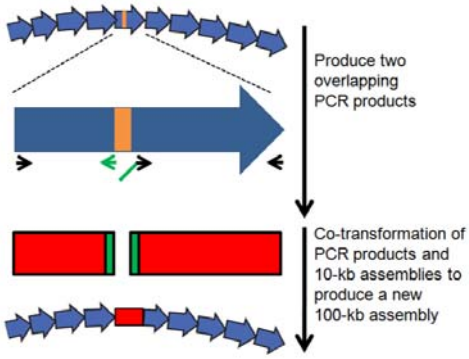
   - **The TREC gene or region deletion method**: Two sequential recombination steps are used. The first step replaces the indicated 450 bp region with the CORE sequence. The second step involves induction of the SCEI endonuclease gene and the cleavage at the SceI site. Homologous recombination at the two repeats (blue arrows) completes the seamless deletion.



   - **The Green Monster process** uses a sexual cycling scheme in yeast. Single-mutant haploids (light green) are mated. Meiotic recombination during sporulation of the mated diploids generates a mixture of 0-GFP cells (white), 1-GFP cells (light green), and 2-GFP cells (dark green). Flow cytometry selects the greenest cells enriched for the 2-GFP cells. This cycle is repeated to generate higher-order multimutants.



- **PCR-based reassembly strategy for deleting genes from the *M. mycoides* genome.** A single gene (orange) is deleted from an existing 10- kb assembly (blue arrow) to produce a new 10-kb assembly (red rectangle) that is directly assembled with nine other 10-kb assemblies to produce a new 100-kb assembly.



Results and Discussion

TOP DOWN APPROACH

Transposon mutagenesis has been completed. Sequencing of the Tn4001 transposon study found approximately 3000 unique insertion sites. The Tn5 study produced 10,902 unique insertion sites, with 754 genes disrupted and 160 not hit by the transposome. The number of hit genes is high because there is extensive functional redundancy. For example, there are two rRNA operons and only one is necessary for viability. The interpretation of the transposon insertion maps is not straightforward, and we believe that the Tn5 data is more reliable because Tn5 cannot make secondary movements into new locations. The transposon mutagenesis data was used to construct the global transposon map contained in attachment A. We will continue to experiment with new Tn5 transposome designs that may help to improve data reliability.

Targeted, stepwise gene deletion is ongoing and progress to date is shown below:

	Size
M. mycoides wild type	1089 kb
M. mycoides JCVI-syn 1.0	1079 kb
M. mycoides JCVI-syn 1.0 – 6RM (12 genes, 17 kb)	1062 kb
M. mycoides JCVI-syn 1.0 – 6RM (12 genes, 17 kb) – 6 IS (12 genes, 9 kb)	1051 kb
M. mycoides JC syn1.0 – 6RM(12 genes, 17 kb) – 6 IS (12 genes, 9 kb) – ICE (44 genes, 71 kb)	980 kb
M. mycoides JC syn1.0 – 6RM(12 genes, 17 kb) – 6 IS (12 genes, 9 kb) – ICE (44 genes, 71 kb) – D5 deletions (52 kb)	928 kb

We plan to continue removing the large clusters, testing for viability at each step. After that, small clusters and individual non-essential genes will be removed to arrive at the minimal genome.

BOTTOM UP APPROACH

Two versions of a minimal genome were designed; one by the team based in Rockville, MD and one by the San Diego, CA team. The first four 1/8 molecules were taken from the Rockville team and the last four 1/8 molecules were taken from the San Diego team for a total size of 483 kb. We are following a four stage assembly strategy:

16,000 oligonucleotides (70 bases each)
↓
370 stage-1 assemblies (1.4 kb)
↓
74 stage-2 assemblies (6.7 kb)
↓
8 stage-3 assembles (50-75 kb)
↓
483 kb Minimal Genome

All of the 370 first stage assemblies and all of the second stage assemblies have been completed. However, many of the second stage assemblies have mutations, which are being corrected by site directed mutagenesis. Following complete sequence verification of the stage-2 assemblies, 1/8 molecules will be assembled and tested for functionality by combining with 7 functionally verified pieces.

Conclusions

The Task 1 deliverables from the Statement of Work, shown below, have been completed:

1. Tn5 transposon insertion map: The global transposon mutagenesis map is contained in Attachment A for reference.

2. *M. mycoides* JCVI-syn1.0 with deletions of 6 restriction systems, 6 IS elements, and the ICE element and 7 other large gene clusters: These regions were deleted and the reduced genome was transplanted and found to be viable, with a doubling time of approximately 60 minutes.

Planned Activities for the Next Reporting Period

We have initiated work on Task 2 – the deletion of up to 27 large gene clusters using TREC, synthetic reconstruction and combinatorial methods. We will continue this effort through the end of Phase I, Year 1. We will also continue to work on the "Bottom Up" approach to synthesize the two minimal genome designs. 1/8th genome molecules will be tested for functionality.

Program Financial Status

	Planned Expend	Actual Expend (Cumulative to Date)	% Budget Completion	At Completion	Latest Revised Estimate	Remarks
Task 1	$604,108.80	$151,358.84	N/A*	N/A*	$300,000	N/A
Cumulative	$1,175,389.00	$151,358.84	12.8%	N/A	$1,175,389.00	N/A

*Expenses for the month of July are not yet available due to the timing of this report.

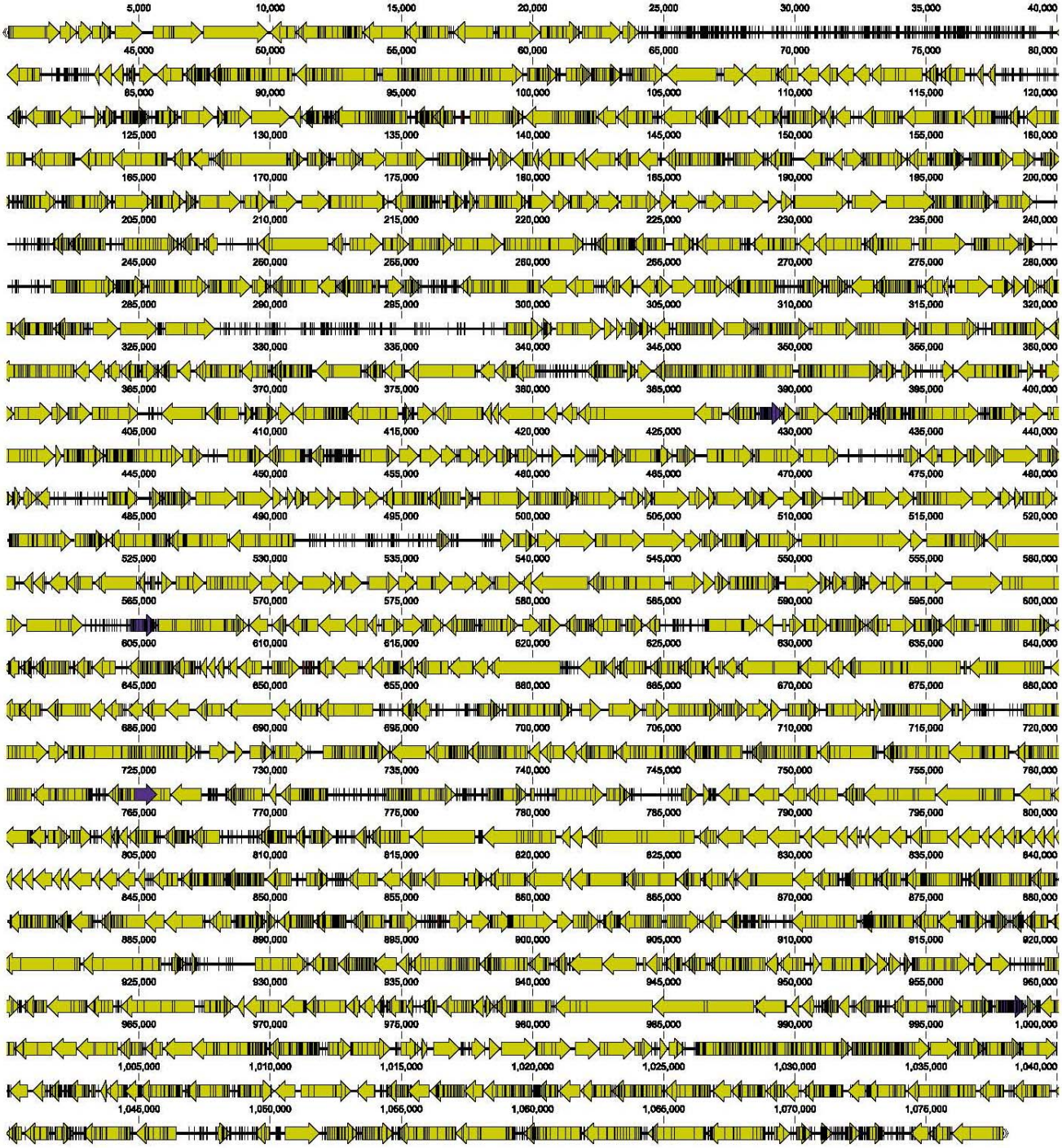
There is no management reserve or unallocated resources.

Based on the currently authorized work:

- Is current funding sufficient for the current fiscal year? Yes

- What is the next fiscal year funding requirement at current anticipated levels? The budgeted amount for Year 2 of the project is $1,214,151.00.

- Have you included in the report narrative any explanation of the above data and are they cross-referenced? Not applicable; current funding is sufficient for the current fiscal year.



Combined map of *Mycoplasma mycoides* JCVI-syn1.0, depicting transposon insertions. Yellow arrows correspond to genes, purple corresponds to watermark sequences, and black lines indicate transposon insertions.